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Trends in Microbiology

Forum

Higher-order microbiome interactions and how to find them

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The complexity of microbial communities suggests prevalent interactions involving more than just pairs of species. These so-called higher-order interactions may reveal new molecules that enable bacteria to deal with complex environments. This forum article discusses how higher-order interactions can be detected and why molecular biologists might care.

Introduction

Bacteria interact with one another and their environment through molecules that are the products of genes. The study of molecules induced by microbial interactions spans from the historic discovery of penicillin (mold–bacteria interactions) to current innovations with Cas9 (bacteria–phage interactions). Microbial communities often contain tens to thousands of strains, each with its own biochemical potential. Most of the molecules of microbial interactions are unknown, and some can aid humanity. However, just as the different cell types of a human liver utilize distinct sets of genes to perform their functions, bacteria use specific genes in specific contexts, meaning that many molecules remain unexpressed in laboratory monocultures. Context-dependence is crucial for organisms in nature because conditions are constantly changing and evolution has undoubtedly adapted genomes to deal with multiple contexts, for example, the diauxic shift. An important question then is: how do organisms deal with a wide range of contexts? Experimentally, how can we detect important contexts? The framework of epistasis, which has been applied at the genomic scale to infer genetic pathways, can be adapted to the scale of microbial communities to detect interactions [1]. These interactions in turn define the contexts that are important for turning on unstudied genes, allowing targeted approaches to uncover new molecules of consequence, including environmental sensors, information processors, and effectors such as antibiotics.

Does community complexity require new biology?

How do organisms sense a complex environment? How do they decide on the optimal response? And how do they dynamically regulate their behavior [2]? Answering these fundamental questions has revealed genetic switches, recombinases, persister cells, and the stringent response. Do the needs of a species adapting to its environment become more complex as the number of neighboring species increases? For instance, can a limited number of receptors recognize an exponentially large set of conditions? Do cells handle this complexity with higher-level regulatory programs? Or do they simply deal with the unpredictability of their environment through ecological mechanisms such as bet-hedging [3]? Evolution has often crafted specific molecules to deal with specific circumstances. Encoding a separate sensor (e.g., a two-component system) for each different environmental factor (e.g., nutrients, stressors, toxins, competitors) might be sufficient in laboratory monocultures but cannot account for the level of complexity found in nature, when these environmental factors (100s of them) come in complex combinations (~2^{100}) [4]. How do bacterial information-processing systems deal with such complex environments? How does a cell tune into its own quorum sensing system and tune out its neighbors when 100 species coexist and the signals overlap imperfectly? Understanding cell behaviors requires a precise understanding of the contexts that elicit the behaviors.

Experimental approaches to detect interactions in communities use combinatorics

Many interactions occur only in a specific context of other organisms and environmental conditions [5,6] (Figure 1). Exploratory work to map the extent of context dependence using complete combinatorial experimental designs [6,7] has been enabled by new high-throughput techniques [8], where a given set of biological phenotypes are measured under all possible permutations of species. This has also spurred the development of new theory, with a goal of predicting the complexity of microbial communities [9]. We classify two main types of experiments to explore microbial interactions. Omics-style approaches, which are covered in other reviews, try to characterize, for example, the genes expressed for each organism in a community. Combinatorial experiments typically measure an explicit phenotype with all possible permutations of organisms (Figure 2). Epistatic genetic screens use this approach to define the genetic pathways of cells. Here, we apply the epistatic screen approach to microbial ecology. A mathematical model of epistasis is needed to calculate the interactions.

Applying the genetic epistasis concept to detect microbial interactions

The fundamental concept behind all epistasis frameworks is the null hypothesis of additivity between genetic elements: if we understand how each element works independently, can we predict how they will work in combination? Statistically significant deviations from the additive prediction are interactions. When genetic epistasis is used to determine whether two genes are in the same pathway, the basic question is whether a double-mutant phenotype can be predicted by adding the two corresponding single

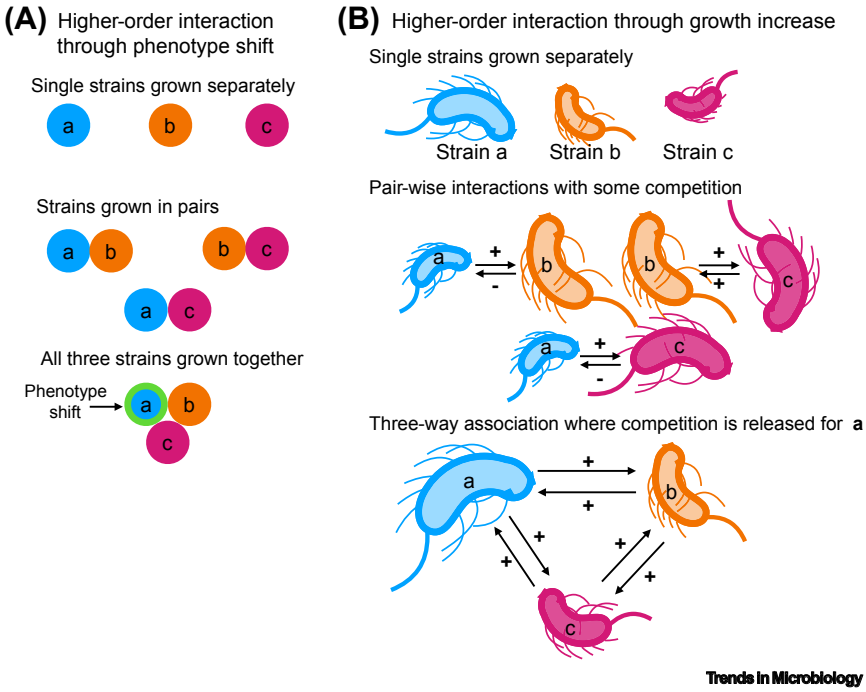

Figure 1. Two hypothetical microbial higher-order interactions. (A) This highly simplified cartoon symbolically illustrates the principle of a higher-order interaction. Interactions can be detected through expression of a novel phenotype, such as when Actinobacteria are induced to produce an antibiotic by another strain [15]. The cartoon depicts a three-way interaction where strain 'a' produces a new colony color when both 'b' and 'c' are present. (B) Higher-order interactions can be detected through changes in abundance of the individual strains ('a', 'b', and 'c') grown separately, in pairs, and all three together. The size of the cartoon cells indicates the resulting population sizes. A three-way interaction is demonstrated because the blue strain grows really well only in the presence of the orange and pink strains. Why does this happen? Two possible scenarios are (i) nutritional competition and provisioning versus (ii) signaling. In scenario (i), the pink and orange cells each compete with the blue strain for one nutrient but also provide a different essential nutrient. Overlaps in their provisioning and competition provides complete nutrition for blue only when both are present. In scenario (ii) blue waits to grow until it receives a combined signal from orange and pink despite having sufficient nutrition to grow.

mutant phenotypes, with the null hypothesis that the phenotypes are additive and thus the genes are in separate pathways. To conceptualize epistasis between bacterial strains, consider each bacterial genome as a genetic locus for epistasis. As an analogy, the abundance of a species corresponds to the expression of a set of genes from the genome. Mathematically, the same approach has been applied to understand community interactions in ecosystems, with the loci being animals and plants [10]. The key result that the analysis provides is a defined context (in terms of which other species are present) that drives an interaction between a pair of species.

Higher-order interactions

The frameworks to detect epistasis have been extended to three or more species in what are called higher-order interactions (reviewed in [1]). A higher-order interaction means that three or more strains are necessary for the interaction, for instance, a third species can change the interaction between two species (Figure 1B). This is similar to how the genetic background can change the interaction between two genes [11]. Interactions can be direct, for example, contact-dependent killing of another organism or production of a targeted antibiotic; interactions can also be indirect, for example, changing the pH of the environment, consuming a shared resource, or changing host physiology (Figure 2). The types of interactions that are detected depend on the phenotypes analyzed. For instance, an example of a higher-order interaction phenotype would be the production of a pigment in one species only when two specific other species are also present (Figure 1A). For molecular biologists, this simple paradigm can be adapted to interaction discovery without a complex mathematical framework. To discover new molecules, including secondary metabolites, environmental sensors, and cellular decision machinery, traditional bioassays including microbial growth inhibition and switches in colony morphology or color can be adapted to the context of a microbial community (Figure 1A).

Experimental considerations

A true higher-order interaction is established by analyzing all possible combinations of the interacting organisms [12] (Figure 1). Thus, the number of conditions scales as 2^(number of species) minus one (Figure 2). In practice, five species is convenient because three replicates of all combinations ($2^5 = 32$) can be run together on one 96-well plate.

An important consideration is: which species are combined? A simplistic approach, in which stock center isolates are combined, potentially misses a vast amount of biology because the stock center strains did not evolve together in a community. Isolating wild strains from natural communities circumvents this pitfall but also brings along additional constraints in terms of genetic tractability and developing appropriate experimental conditions to elicit interactions.

Many technical factors can influence the outcome of a combinatorial experiment

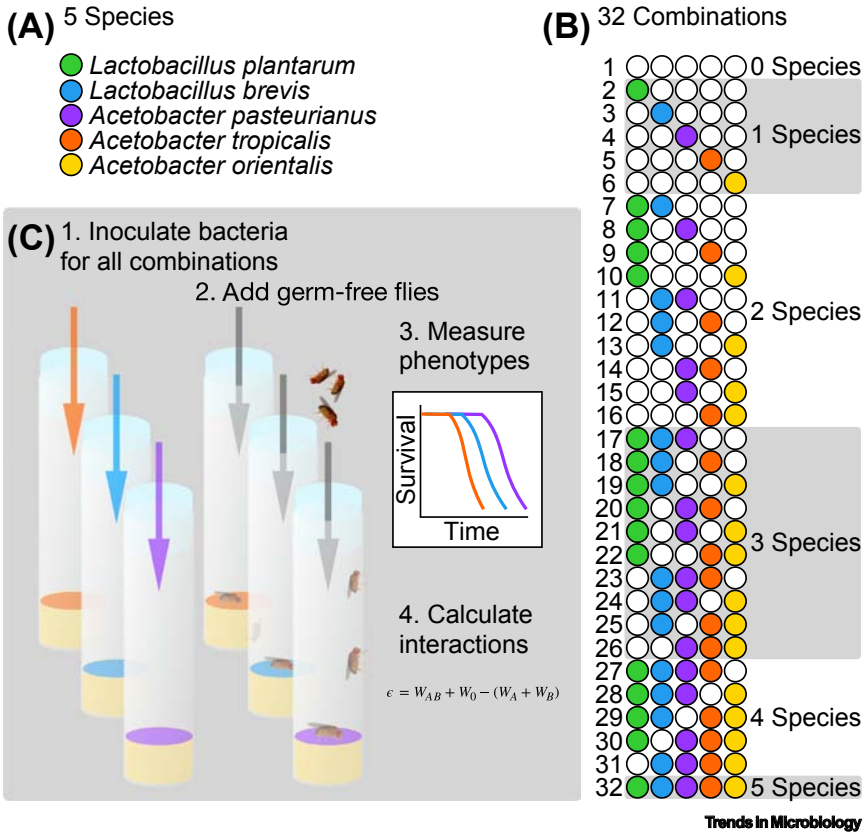

Figure 2. Cartoon of a combinatorial experiment from the fly gut microbiome to detect interactions that affect the host. (A) Five bacterial strains are naturally present in the Canton-S lab fly. (B) Making each combination of the five strains produces 32 different microbial treatments. (C) Each of the combinations is inoculated onto germ-free fly food. Then germ-free flies are added. Phenotypes, for example, survival and fecundity, are measured. Finally, the phenotypes for each combination are input into a mathematical model to extract the interactions.

and can also be used to narrow the candidate mechanisms. One important factor is how the abundances of individual species vary across combinations. Abundance is important to track to make sure that all inoculated species are present. Moreover, if a species' abundance correlates (or anticorrelates) with a measured phenotype, this can suggest hypotheses about causation. CFUs plating is often used to quantify viable cell populations. Alternatively, 16S amplicon sequencing or qPCR can be paired with an internal reference standard. Another important consideration is how dynamics contribute to the phenotype. For instance, can a lag time in growth explain a lower abundance at the time of quantification? Could such a delay reduce the apparent phenotype? Quantifying the dynamics can potentially aid in determining the mechanism for an interaction. For instance, spatiotemporal dynamics are useful to consider because interactions through diffusible molecules can be differentiated by comparing solid media versus well-mixed liquid culture [13].

What higher-order interactions we might find

In the organs and tissues of higher eukaryotes, distinct cell types coordinate their physiology to produce higher level functions, for example, detoxification, fat redistribution, and immunity by the liver. Microbial communities may have similar higher-level functions that require coordination between multiple cells types. For example, multicellular biofilms with conserved taxonomic makeup and physical structure recur, for instance, in oral microbiomes or in wastewater treatment systems. How microbial cells coordinate their physiology to develop these complex structures remains poorly understood. Interactions could be coordinated through new quorum molecules, nutritional cues, or higher-order signal logic, such as AND, OR, XOR, and NOR gates that allow cellular decision-making in complex environments [14]. Higher-order interaction screens can help to define the natural contexts for such genes, leading to new molecular discoveries.

Declaration of interests

The authors have no interests to declare.

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